Phytology

Molecular characterization of the genus *Cousinia* Cass. (Asteraceae) in Iraq utilizing RAPD markers

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Abstract
The research reported involves the characterization of seven species belonging to the genus *Cousinia* Cass. These species were collected from several locations in Northern Iraq. The study was conducted from 2021 to 2022 at the College of Science, University of Tikrit, located in Tikrit, Iraq. The variation in genetic relations within the species were determined using the random amplification polymorphism DNA (RAPD) technique. The statistical tool Numerical Taxonomy and Multivariate Analysis System (NTSYSpc. 2.10e) was used for this purpose. Amplifications were achieved using 11 random primers, as confirmed by agarose gel electrophoresis. The utilization of RAPD primers resulted in the production of 512 random bands, enabling the differentiation of *Cousinia* species from one another. The degree of genetic similarity among the studied species of *Cousinia* varied from 57.89% to 75.26%. The least genetic similarity, observed at 57.89%, was noted between *Cousinia aintabensis* and *Cousinia stenocephala*. On the other hand, the greatest degree of similarity of 75.26% was found between *Cousinia kopi-karadaghensis* and *Cousinia odontolepis*. The dendrogram displayed three primary clusters, determined by the genetic distance values. The third cluster further subdivided into three separate groupings. RAPD has demonstrated its efficacy as a method for investigating the degree of relatedness between species.

Keywords: *Cousinia*, Asteraceae, Molecular, RAPD Markers, Iraq

Introduction

The genus *Cousinia* Cass. belongs to the tribe Cardueae in the family Asteraceae and consists of around 700 species. It is part of the *Arctium-Cousinia* complex, which is currently recognised as a distinct subtribe called Arctiinae (Susanna *et al.* 2006; Susanna & Garcia-Jacas 2007; López-Vinyallanga *et al.* 2009; Herrando-Moraira *et al.* 2019) [24, 23, 14, 11]. The genus *Cousinia* comprises about 400 species in Southwest Asia, with the greatest concentration of species found in the Flora Iranica region. Among these species, 379 are exclusive to this area. The distribution of these species is limited to hilly regions in Iran, Afghanistan, and Turkmenistan (Rechinger, 1986) [20]. The genus *Cousinia* is among the most extensive genera in the Cardueae tribe of the Asteraceae family. The Irano-Turan realm is habitat to approximately 300 out of the total of over 600 species. The genus ranks fifth in the generic floristic spectrum of the Irano-Turkestanian region (Islamov, 2021) [13]. Iraq and Turkey possess 17 and 8 species, respectively. In contrast, Iran contains a total of 77 distinct groups of organisms, with 66 of them being unique to Iran. Additionally, Iran serves as the central hub for the variety of this specific region's inhabitants (Huber-Morath 1972; Attar and Ghahreman, 2006) [12, 4]. There are currently approximately 24 species of the *Cousinia* genus in Iraq (Ghazanfer *et al.*, 2019) [10]. The taxonomy of the genus *Cousinia* is complex and contentious due to its significant morphological variety (Atazadeh *et al.*, 2020) [3]. Given the dispute around the number and classification of *Cousinia* species, the initial task in researching the genus is to ascertain and define the presumed species. Determining the boundaries between species can be challenging when there are similarities in their morphological characteristics (Wiens, 2007) [25]. The systematic identification has been determined by combining morphological and genetic data (Minaeifar *et al.*, 2011; Eftekharian *et al.*, 2018) [16, 9]. Various molecular markers have been employed in plant taxonomy and phylogeny. Among these, random amplification polymorphism DNA (RAPD) has demonstrated efficacy in distinguishing species, variations, ecotypes, and even genotypes within a single species (*Sheidai et al.*, 2013; Safaei *et al.*, 2016) [22, 21]. The RAPD technique is a straightforward method that involves enzymatically amplifying DNA using randomly selected short primers. It needs minimal technological resources and enables rapid acquisition of diverse genomic DNA fragments, offering a high degree of polymorphism (Dilipan *et al.*, 2017) [8]. The objective of this research was to ascertain the genetic connections between various *Cousinia* species gathered from different regions of Iraq. Additionally, a DNA-based RAPD-PCR technique was employed to clarify and settle the uncertain and disputed classification of these species, which used to depend on traditional morphological characteristics.

Materials and methods

Plant material

Leaf samples were obtained from seven species of the *Cousinia* genus in various regions of Iraq. The vouchers from the examined plants have been stored in the National Herbarium of Iraq. Multiple publications have been utilized to categories the

plant specimens being examined (Post, 1933; Davis, 1975; AL-Rawi, 1964; Ghazanfer *et al.*, 2019) [19, 7, 1, 10]. The relevant information is provided in Tab 1.

Table 1: List of the species and regions currently studied

Species	Locality	Elevation (m)
Cousinia stenocephala Boiss.	Tasloja, Altun Kupri, Dokan, Qara-Hanjer	300-877
Cousinia macrolepis Boiss. & Hausskn.	Pira-Magrun and Dokan	708-1465
Cousinia aintabensis Boiss. & Hausskn.	Shaqlawa and Dohuk.	347-1033
Cousinia pergamacea Boiss. & Hausskn.	Said Sadaq and Penjwin	1620-2037
Cousinia kopi-karadaghensis Rech. Fil.	kupi-Qara-dagh	1534-1842
Cousinia algurdina Rech.f.	Al-Sulaymaniyah and Qandil region.	1300-2450
Cousinia odontolepis DC.	Zawita, Sirsenq, and Amadiya,	800-1400

DNA extraction

Leaves of the specimens belonging to the genus *Cousinia* were gathered and genomic DNA extracted utilizing the GENEAID plant min kit, Bioneer, Korea. The fresh leaves (4g) for each sample are pulverized into a powder using liquid nitrogen a pestle and mortar. Transfer 100 µl of GPX1 solution to the powdered leaf tissue using a tiny pestle for grinding within Eppendorf tubes. 30 µl of proteinase k solution and 200 µl each of GPXI and GPI are added to the mixture from the previous stage. The mixture is then well stirred by vertexing. The combination is transferred to the water bath and maintained at a temperature of 600°C for a duration of 1 hour. Add 100 µl of GP2 and stir by vertexing. Subsequently, a filter column was positioned within a 2ml collecting tube, followed by centrifugation at a speed of 1000 revolutions per minute for a duration of 1 minute. Put 700 µl of GP3 into the remaining mixture and spin using a vortex; thereafter, transfer the mixture to a silica column to separate and eliminate non-genomic DNA components while retaining the genomic DNA. The sample was subjected to centrifugation at a speed of 1350 revolutions per minute (rpm) for a duration of 1 minute. The suspended DNA was mixed with 400 µl of washing solution (W1) and then centrifuged at 1350 rpm for 30 seconds. The suspended DNA is mixed with 600 µl of washing buffer and then subjected to centrifugation at 1350 rpm for 30 seconds. The column matrix is subsequently dehydrated using centrifugation at a speed of 1350 revolutions per minute for a duration of 3 minutes. put 50 µl of elution buffer to the DNA suspension and centrifuge at 1350 rpm for 5 minutes.

Analysis of DNA

The RAPD-PCR examination utilized 11 random primers (Company Bioneer, Korea) (Table 2) and was conducted on all *Cousinia* species. The PCR reaction conditions were consistent, except for the annealing temperature, which was modified. The protocol consisted of one cycle of denaturation at 95°C for 5 minutes, followed by 36 cycles of annealing at 94°C for 1 minute, 36°C for 1 minute, and 72°C for 1 minute. The analysis concluded with a final extension step of 2 minutes at 72°C. The DNA samples extracted from the examined species of *Cousinia* were subjected to random amplification processes. Gel electrophoresis was then conducted on an agarose gel, which was subsequently stained with ethidium bromide for a duration of 20-30 minutes. Subsequently, the gel was subjected to UV transillumination to get a precise measurement of the sizes of the DNA molecules that were amplified bands. This was achieved by comparing their positions within the gel to a typical volume guide (DNA Ladder), as represented in Figure 1.

Table 2: RAPD primers of the examination utilized

No.	Primer name	Primer sequence (5'-3')
1	OPD-02	GGACCCAACC
2	OPF-02	GAGGATCCCT
3	OPI-08	TTTGCCCGGT
4	OPF-04	GGTGATCAGG
5	OPF-07	CCGATATCCC
6	OPO-15	TGGCGTCCTT
7	OPF-09	CCAAGCTTCC
8	OPK-10	GTGCAACGTG
9	OPF-11	TTGGTACCCC
10	OPC-04	CCGCATCTAC
11	OPF-13	GGCTGCAGAA

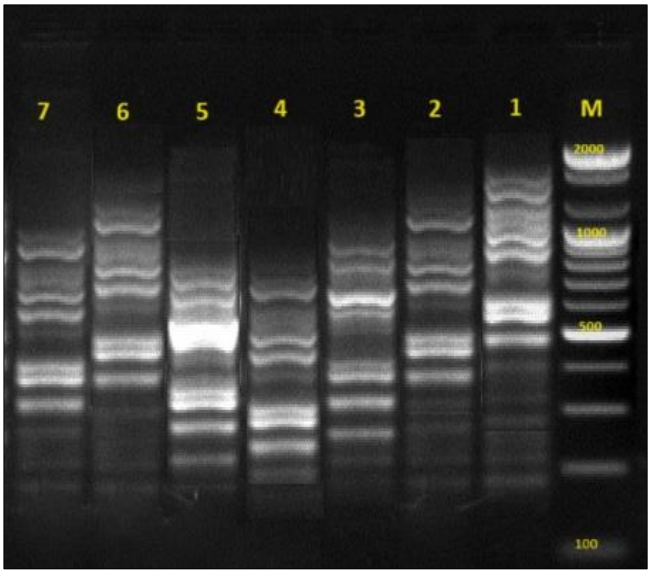

Fig 1: RAPD-PCR reaction using the random primer (OPF-04) of sevens *Cousinia* studied species M (1 Kb DNA Ladder) 1. *Cousinia aintabensis* 2. *Cousinia kopi-karadaghensis* 3. *Cousinia macrolepis* 4. *Cousinia odontolepis* 5. *Cousinia pergamacea* 6. *Cousinia stenocephala* 7. *Cousinia algurdina*.

Allele scoring

The amplified fragments were observed using a UV

transilluminator and documented with a gel recording system (Vilbert Lourmat, Infinity model). The products of amplifying using RAPD analysis were evaluated by assigning a score of 1 for the existence of a band and 0 for the lack of a band for every primer. The pattern of banding of each primer was assessed through visual observations, with only distinct and unequivocal bands being recorded. The amplified bands' size, measured in nucleotide base pairs, was assessed by comparing their migration to a molecular weight marker (DNA ladder from Bioneer, Korea).

Data statistical analysis

Banding patterns acquired from RAPD analysis for each primer were evaluated through visual examination. All of the amplified pieces were regarded as dominant genetic markers. Genetic similarity was determined by calculating pairwise variances in the amplified product for all genotypes, using the presence or lack data. Using this data, a cluster analysis was conducted to determine the correlation between different genotypes**.** The results obtained from the polymorphic pieces were examined using the equation provided by Nei and Li (1979): -

$$\text{Similarity (F)} = \frac{2Mx}{My + Mz}$$

Dissimilarity= 1- F, Mx: Number of identical pieces from genotypes y and z, my: number of genotype y fragments that have been scored, and Mz: number of genotype y fragments that have been scored.

Utilizing the NTSYS 2.10e-PC software programme to compute the genetic similarity distance. The analysis of clusters facilitated the construction of a dendrogram utilizing the UPGMA. The objective of this analysis is to categories all the genotypes into a smaller number of groups or clusters, based on shared characteristics among the genotypes (Aydogan & Yagdi 2012) [6]. The current study aimed to establish standardized protocols for DNA isolation, optimize PCR settings, and create a database of RAPD markers. These objectives were pursued to investigate the genetic variability in specific genotypes of *Cousinia* species studied.

Results and discussion

The RAPD analysis investigated the genetic correlation among the seven species of *Cousinia*. The study employed a total of 11 primers, each containing a distinctive arrangement of nitrogen bases. The specific primers and their corresponding base sequences may be found in Table 2. This study examines the overall number and ratio of polymorphic bands and variations produced by DNA amplification, based on the nitrogen base sequence of the random primers employed for the *Cousinia* species being investigated. The random amplification of DNA yielded band patterns, with a total of 512 bands on the sample genome being recognized by the primers. On average, each primer produced 46.5 bands, out of which 103 were monomorphic, with an average of 9.3 bands per primer. Similarly, there were 409 sites that exhibited polymorphism, with an average of 37.1 variations for each primer. The primer OPF-02 produced the greatest number of bands, with a total of 67, while the primer OPD-02 produced the fewest number of bands, with just 21. The primers on the sample genome identified a total of 26 unique sites, The primer OPF-02 gave the highest number of unique bands, totaling 67, while the primer OPD-02 yielded the lowest number of bands, amounting to only 21. The primers OPF-04 and OPC-04 produced the greatest number of unique bands, with 4, while the primers OPD-02, OPF-07, and OPF-11 produced the fewest number of unique bands, with just 1. These bands were considered to be the most essential diagnostic features used to differentiate between species.

Table 2: Result of the amplification of DNA bands obtained in the examined *Cousinia* species utilizing 11 primers

Primer name	Total no. of bands	Unique band	Polymorphic bands	Monomorphic bands	Bands/Genotype	Polymorphic markers	Pic	Band size
OPD-02	21	1	21	0	3.00	100.00%	0.8767	1100-300
OPF-02	67	2	55	12	9.57	82.08%	0.9894	1600-150
OPI-08	61	2	52	9	8.71	85.24%	0.8932	1800-250
OPF-04	47	4	34	13	6.71	72.34%	0.8276	1500-200
OPF-07	39	1	32	7	5.57	82.05%	0.9766	1400-200
OPO-15	58	2	43	15	8.28	74.13%	0.8132	1900-100
OPF-09	48	2	40	8	6.85	83.33%	0.7978	1700-250
OPK-10	64	3	47	17	9.14	70.14%	0.9277	1300-250
OPF-11	37	1	30	7	5.28	81.08%	0.8996	1650-175
OPC-04	41	4	31	10	5.85	75.60%	0.7789	1450-150
OPF-13	29	2	24	5	4.14	82.75%	0.8689	1700-200
total	512	26	409	103	-			

The primer OPD-02 exhibited unique bands with a molecular size of 550 pb, specifically for the species *Cousinia macrolepis*. The primer OPF-02 displayed two unique bands with a molecular size of 380 pb, exclusively targeting the species *Cousinia pergamacea*, and species *Cousinia aintabensis* at molecular size 730pb. The primer OPF-04 exhibited unique bands with a molecular size of 450, 580, and 950 pb, specifically for the species *Cousinia kopi-karadaghensis*, and the species *Cousinia algurdina* with a molecular size of 640 pb. The primer OPK-10 show three unique bands with a molecular size of 530, 850, and 1150 pb for the species *Cousinia stenocephala*. The smallest value of polymorphic 70.14% was created in OPK-10 primer, while the maximum level of polymorphic 100.00% was produced OPD-

02 primer. The range of the RAPD marker's polymorphic information content (PIC) was from 0.7789 to 0.9894. The maximum PIC value of 0.9894 was obtained with the OPF-02 primer, while the lowest PIC value was observed with the OPC-04 primer. A total of eleven primers were employed to determine the connections among seven genotypes belonging to the *Cousinia* genus. The product bands size ranged from 100 to 1900 bp. Monomorphic bands are bands that have the same mobility in gel electrophoresis, whereas polymorphic bands are bands that appear in different positions on the electrophoresis gel for different samples. The similarity coefficients among *Cousinia* species investigated can be shown in (Fig. 2 & Table 3) utilizing the RAPD fragments to build a dendrogram using the UPGMA process. The 7 species of *Cousinia* were classified into five clusters. Pearce *et al.*, (2005) [18] verified that accessions can be categorized into a single group based on their geographical location or based on their origin and lineage. The genetic similarity between the 7 species of Centaurea varied from 57.89% to 75.26%. The minimal genetic similarity, recorded at 57.89%, was observed between *Cousinia aintabensis* and *Cousinia stenocephala*. On the other hand, the greatest similarity, at 78.54%, was found between *Cousinia kopi-karadaghensis* and *Cousinia odontolepis*, and the other species intertwine together. The species relationship derived from morphological traits aligns with earlier research. For instance, *Cousinia kopi-karadaghensis* and *Cousinia odontolepis* were grouped together since they shared similarities in all characteristics except for the color of the corolla. (Asaadi and Mehregan, 2017) [2] also observed their strong attachment for one other.

Table 3: The similarity coefficient comparisons among the seven *Cousinia* species that were evaluated

Species	*Cousinia aintabensis*	*Cousinia kopi-karadaghensis*	*Cousinia macrolepis*	*Cousinia odontolepis*	*Cousinia pergamacea*	*Cousinia stenocephala*	*Cousinia algurdina*
Cousinia aintabensis	1.0000						
Cousinia kopi-karadaghensis	0.6211	1.0000					
Cousinia macrolepis	0.5842	0.6158	1.0000				
Cousinia odontolepis	0.6368	0.7526	0.6105	1.0000			
Cousinia pergamacea	0.5895	0.6421	0.6579	0.6684	1.0000		
Cousinia stenocephala	0.5789	0.6526	0.6579	0.7421	0.6947	1.0000	
Cousinia algurdina	0.6684	0.7000	0.6316	0.7263	0.6474	0.6789	1.0000

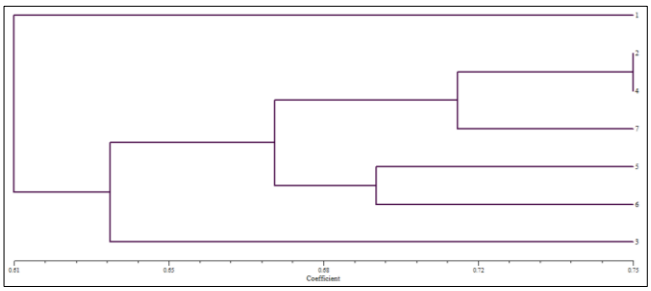

Fig 2: The dendrogram was created using marker RAPD data to illustrate the genetic link among the 7 *Cousinia* species being investigated. 1. *C. aintabensis* 2. *C. kopi-karadaghensis* 3. *C. macrolepis* 4. *C. odontolepis* 5. *C. pergamacea* 6. *C. stenocephala* 7. *C. algurdina*

Both *C. aintabensis* and *C. stenocephala* exhibited Genetic divergence agree with morphological different between it in terms of the number of flowers, the color of the anther tube, receptacle bristles, and head diameter. The study conducted by Ghazanfer *et al.* (2019) [10] also demonstrated the presence of distinct morphological different among these species. The other investigated species of *Cousinia* shown variations regarding their genetic tree compared to their morphological characteristics. The result is in accordance with findings of Lopez-Vinyallanga *et al.*, (2009) [14], since they reported that phenotypic attributes are extremely inconsistent with genetic evidence in *Arctium-Cousinia* complex and regarded phenotypic features homoplasious**.** The RAPD data revealed a genetic similarity between *C. odontolepis* and *C. stenocephala*, as well as between *C. algurdina* and *C. odontolepis*. These species also exhibited morphological resemblances. The RAPD results indicated a strong similarity between *C. algurdina* and *C. kopi-karadaghensis*, which is consistent with the findings from the morphological data. The findings closely align with the morphological taxonomic treatment of the *Cousinia* genus, as proposed by (Attar and Djavadi, 2010; Asaadi and Mehregan, 2017; Ghazanfer *et al.*, 2019) [5, 2, 10]. The similarity coefficient in Table 3 indicated the genetic differentiation between *C. aintabensis* and *C. stenocephala*, as well as between *C. pergamacea* and *C. aintabensis*. in spite of these species also revealed morphological resemblances. Several factors were proposed to explain this discrepancy between genetic and phenotypic analyses: The genus *Cousinia* has a large number of species, characterized by homoplasious morphological characteristics, which are the result of evolutionary convergence (Zhang *et al.*, 2015) [26].

Conclusion

the current study revealed that the morphological features and RAPD molecular data are helpful in correctly recognizing and genetic relationship among the *Cousinia* examined species. Both the qualitative and quantitative morphological traits are crucial and appropriate for recognizing a species within the genus *Cousinia*.

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